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CHOLESTEROL (LIQUID) REAGENT SET

INTENDED USE
For the quantitative determination of total cholesterol in serum.

INTRODUCTION
Cholesterol is a fatty substance found in blood, bile and brain tissue. It serves as a precursor to bile acids, steroids and vitamin D. The determination of serum cholesterol is a major aid in the diagnosis and classification of lipemias.[1] Other conditions such as hepatic thyroid diseases influence cholesterol levels.[2]

Enzymatic methods have replaced older methodologies involving cholesterol esterase, oxidase, and trinders color system. Allain et al. developed a total enzymatic technique in which hydrogen peroxide during the oxidation of cholesterol is used in conjunction with peroxidase, 4-aminoantipyrine and phenol to form a quinoneimine dye.[3] This reagent employs p-hydroxy benzene sulfonic acid (p-HBS), in place of phenol to produce a quinoneimine dye with greater absorbance at 520 nm and a surfactant to facilitate the completion of reaction.
The results obtained from this replacement are equal to the results obtained based on the Abell-Kendall cholesterol reference method which is recommended by the CDC.[6]

PRINCIPLE
The enzymatic reaction sequence employed in the assay of cholesterol is as follows:

$$\text{Cholesterol Esters} \xrightarrow{\text{C. Esterase}} \text{Cholesterol + Fatty Acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{C. Oxidase}} \text{Cholesten-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{4-Aminoantipyrine} + \text{p-HBS} \xrightarrow{\text{H. Peroxidase}} \text{Quinoneimine} + 2\,H_2O \quad \text{(red dye)}$$

Cholesterol esters are hydrolyzed to produce cholesterol. Hydrogen peroxide is then produced from the oxidation of cholesterol by cholesterol oxidase. In a coupled reaction catalyzed by peroxidase, quinoneimine dye colored red is formed from 4-aminoantipyrine, p-HBS and hydrogen peroxide. The absorption at 520 nm of the solution of this dye is proportional to the concentration of cholesterol in the sample.

REAGENT COMPOSITION
Cholesterol (liquid) reagent set contains the following:
1. Cholesterol Reagent:
   4-Aminoantipyrine 0.6 mM, Sodium Cholate 8.0 mM, Cholesterol Esterase ≥ 150 U/L, Cholesterol Oxidase ≥ 150U/L, Horseradish Peroxidase ≥ 1,200 U/L, p-Hydroxy benzene sulfonate 20 mM, Buffer 125 mM, pH 6.8, non-reactive ingredients.
2. Cholesterol Standard (Liquid): 200 mg/dl.
   This standard was made with materials traceable to Standard Reference Material available from the National Institute of Standard and Technology.

REAGENT PREPARATION
All reagents come in a ready-to-use form. No preparation is necessary.

WARNINGS AND PRECAUTIONS
1. For *in vitro* diagnostic use.
   CAUTION: In vitro diagnostic reagents may be hazardous. Handle in accordance with good laboratory procedures which dictate avoiding ingestion, and eye or skin contact.
2. Specimens should be considered infectious and handled appropriately.

REAGENT STORAGE AND STABILITY
Store the reagent set at 2 - 8°C.

REAGENT DETERIORATION
The reagent should be discarded if:
1. Turbidity has occurred; turbidity may be a sign of contamination.
2. The reagent fails to meet linearity claims or fails to recover control values in the stated range.

SPECIMEN COLLECTION
1. Test specimens should be serum and free from hemolysis.
2. Cholesterol in serum is reported stable for seven (7) days at room temperature (18 - 25°C) and six (6) months when frozen and properly protected against evaporation.[4]

INTERFERING SUBSTANCES
Anticoagulants such as fluoride and oxalate will result in false low values.[5] The test is not influenced by hemoglobin values up to 200 mg/dl or by bilirubin levels up to 10 mg/dl. Interference from grossly icteric and heavily hemolyzed specimens is correctable by use of a serum blank.[5]

MATERIALS PROVIDED
1. Cholesterol Liquid Reagent
2. Cholesterol Standard

MATERIALS REQUIRED BUT NOT PROVIDED
1. Spectrophotometer capable of measuring absorbances at 520 nm
2. Test tubes/rack
3. Accurate pipetting/measuring devices
4. Timer
5. Heating block (37°C)

GENERAL INSTRUCTIONS
The reagent for Cholesterol is intended for use either as an automated procedure on chemistry instruments or as a manual procedure on a suitable spectrophotometer.

AUTOMATED PROCEDURE
Please refer to appropriate application manual available.

MANUAL PROCEDURE
1. Label test tubes: blank, standard, control, patient, etc.
2. Pipette 1 .0 ml of reagent to all tubes and pre-warm at 37°C for at least two (2) minutes.
3. Add 0.01 ml (10 µl) of sample to respective tubes, mix and return to 37°C.
4. Incubate all tubes at 37°C for ten (10) minutes.
5. Zero spectrophotometer with the reagent blank at 520 nm (Wavelength range: 500 - 550 nm).
6. Read and record absorbances of all tubes.

* *TC MULTI-PURPOSE CALIBRATOR MAY BE USED TO REPLACE STANDARD.*

NOTE:
If the spectrophotometer being used requires a final volume greater than 1.0 ml for accurate reading, use 0.025 ml (25µl) of sample to 3.0 ml of reagent. Perform the test as described above.

CALIBRATION
The procedures are calibrated with the standard solution, which is included with each series of tests. Its absorbance is used to calculate the results. It is recommended to establish a linearity curve up to 500 mg/dl with other available commercial standard solutions to verify the performance of instruments and reagents.

LIMITATIONS
The reagent is linear to 500 mg/dl.
1. Samples with values above 500 mg/dl should be diluted 1:1 with isotonic saline and re-run. Multiply final results by two (2).
2. Grossly lipemic serums require a "sample blank." Add 0.02ml (20 µl) of sample to 2.5ml saline, mix and read the absorbance against water. Subtract this value from the patient absorbance to obtain the corrected reading.

CALCULATIONS
(A = Absorbance)

A (patient) / A (standard)	× Concentration of standard = (mg/dl)	Concentration of patient (mg/dl)

Example:
A (patient) = 0.40, A (standard) = 0.32, Concentration of standard = 200 mg/dl.

$\frac{0.40}{0.32} \times 200 = 250$ mg/dl

QUALITY CONTROL
1. It is recommended that high and low values of cholesterol controls be included in each set of assays. Commercially available control material with established cholesterol values may be used for quality control. The assigned value of the control material must be confirmed by the chosen application. Failure to obtain the proper range of values in the assay of control material may indicate either reagent deterioration, instrument malfunction, or procedural errors.
2. It is recommended that CDC or NIST certified reference material is included in the assay to assure the performance of the test.

EXPECTED VALUES[6]
It is strongly recommended that each laboratory establish its own normal range.

RISK CLASSIFICATION	TOTAL CHOLESTEROL IN BLOOD (mg./dl)
Desirable	< 200
Borderline high	200-239
High	≥ 240

PERFORMANCE CHARACTERISTICS
1. *Linearity*: 500 mg/dl.
2. *Sensitivity:* An absorbance change of 0.001 at 520 nm corresponds to 1 mg/dl under the stated condition of this assay system.
3. *Comparison*: A comparison between this procedure and one which is certified by the Center for Disease Control (CDC) and the National Cholesterol Education Program (NCEP) based on human samples assayed both by the Abell-Kendall's method and by the method being certified produced a regression equation of $y = 0.94 x - 2.80$ (N= 47) with a coefficient of correlation of 0.99.
4. *Precision:*

Within Run		
Mean (mg/dl)	S.D.	C.V. (%)
150	9.7	6.5
124	8.8	7.0

Run-to-Run		
Mean (mg/dl)	S.D.	C.V. (%)
142	7.4	5.2
114	6.1	5.3

5. *Specificity:* Cholesterol Oxidase is not totally specific for cholesterol. Other analogs of cholesterol (dihydrocholesterol, 7-dehydrocholesterol, 2 0 hydroxycholesterol, etc.) are also oxidized. These analogs do not normally occur in any appreciable amounts in serum.

REFERENCES

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